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Expression of apolipoprotein E during nerve degeneration and regeneration

(high density lipoprotein/nerve repair/macrophages/Schwann cells/cholesteryl esters)

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ABSTRACT **A 37-kDa glycoprotein has been described recently, whose synthesis is dramatically increased after injury of the rat sciatic and optic nerves. Cells in the nerve sheath, distal to the site of injury, produce and secrete large amounts of this protein, so that by 3 weeks after injury, it represents 2–5% of the total soluble extracellular protein in the regenerating sciatic nerve sheath, although it fails to accumulate in damaged optic nerve. Results presented here reveal extensive homology between the 37-kDa nerve injury-induced protein and a well-studied serum protein, apolipoprotein E (apoE), that is involved in lipid and cholesterol metabolism and that has been shown recently to be present in adult and developing rat astroglia. Both proteins have identical isoelectric focusing points and similar molecular masses. Antibodies raised against the 37-kDa protein recognize apoE and anti-apoE serum crossreacts with the 37-kDa protein. Sequence data for two 14 amino acid stretches of the 37-kDa protein match identical regions of apoE. These data suggest that the 37-kDa protein is identical to serum apoE and that it could have similar functions to the latter. In the nervous system, for example, it may be involved in the mobilization and reutilization of lipid in the repair, growth, and maintenance of myelin and axonal membranes, both during development and after injury.**

Axons regenerating after a nerve injury grow through an extracellular environment elaborated by glia and other non-neuronal cells of the distal nerve stump. A century of clinical and experimental observations has demonstrated the profound influence of the distal nerve stump on successful nerve repair (1, 2). When a peripheral nerve is injured, a series of striking changes occurs in the distal nerve stump. Axons cut off from their cell bodies degenerate, Schwann cells proliferate and align themselves into longitudinal "Büngner bands," while blood-borne macrophages accumulate in the distal sheath to assist in removal of axonal and myelin debris. At the molecular level, one of the most prominent effects of injury is a dramatic increase in the synthesis of an acidic 37-kDa protein that is secreted into the extracellular space (3, 4).

Synthesis of this 37-kDa protein increases more than two orders of magnitude over the first 2 weeks after nerve injury, and the secreted protein accumulates in sufficient abundance to account for 2–5% of the total soluble protein in the extracellular space (3). This protein is also synthesized in neonatal nerves and in injured adult optic nerves and spinal cords; however, it fails to accumulate in these injured central nervous system (CNS) sites (3, 5, 21). The functional significance and identity of this injury-induced protein have remained obscure. Here, we present evidence that the 37-kDa protein induced in injured peripheral nerves is identical to apolipoprotein E (apoE), a serum protein normally involved in lipid transport and metabolism (6, 7). This identity suggests possible roles for the 37-kDa protein in development, degeneration, and regeneration in the nervous system.

MATERIALS AND METHODS

Surgery. Sciatic and optic nerve crushes and labeling and collection of sheath cell proteins were according to Skene and Shooter (3).

For *in situ* or *in vivo* labeling of proteins, the initial surgery was as before. After 1 week, the sciatic nerves were re-exposed under anesthesia, and 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine with 0.05% bromphenol blue as a tracer in a total volume of 5 μl was injected into the subendoneurial space of the nerve with a 31-gauge needle. Injections were done 1–2 mm distal to the original site of injury and in a comparable region in uninjured control nerves. After 24 hr, nerves were removed and immediately frozen at −80°C in a dry ice/ethanol bath. To collect the labeled soluble proteins, nerves were homogenized in a solution of 10 mM Tris·HCl at pH 7.5 containing 2% $NaDodSO_4$, 5 mM EDTA, and 1 mM dithiothreitol. Supernatants of this homogenate, collected after a 10-min, 12,000 × *g* spin, were then analyzed.

Antibody Production and Detection. Rabbits were inoculated by injecting 250 μg of 37-kDa protein purified from 3-week conditioned medium (for details, see ref. 8). The protein was suspended in Freund's complete adjuvant (Difco) and then injected subcutaneously at eight sites along the back of a New Zealand White rabbit. After 1 month, three subsequent booster injections every other week with 50 μg of protein in Freund's incomplete adjuvant yielded antiserum of high titer directed against the native and denatured 37-kDa proteins. Protein blots or electrophoretic transfer analyses were done essentially according to Towbin *et al.* (9), with the primary antibody at dilutions of up to 1:100,000. Bound primary antibody was visualized with peroxidase-labeled second antibody (Vector Laboratories, Burlingame, CA). Controls with nonimmune serum were done at 1:1000 dilution.

For immunoprecipitations, 200 μl of labeled conditioned medium was combined with 10 μl of whole antiserum to the 37-kDa protein and brought to a final volume of 1 ml with a solution of 0.15 M NaCl, 25 mM Tris·HCl (pH 7.4), 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA and rotated for 1 hr at 4°C. Antigen–antibody complexes were pelleted with protein A-Sepharose CL-4B

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Abbreviations: HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); apoE, apolipoprotein E; PNS, peripheral nervous system; CNS, central nervous system; FITC, fluorescein isothiocyanate.

(Sigma), analyzed on 10% polyacrylamide/$NaDodSO_4$ gels, and fluorographed as described below.

Astrocyte-enriched cultures derived from 3- to 5-day-old rat brains were stained according to Raff *et al.* (10) with the following modifications. Before fixation in ethanol/acetic acid, cultures were incubated in 10 μM monensin (Sigma) for 3 hr at 37°C in culture medium. Anti-37-kDa antiserum at 1:200 dilution was applied, followed by application of fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit serum (Cappel, St. Louis, MO; 1:50) and photography with a Zeiss microscope at a final magnification of 250×.

Amino Acid Sequence Analysis. Sequence analysis of the purified 37-kDa protein was essentially as described by Hunkapiller and Hood (11).

Sample Preparation and Electrophoresis. One-dimensional polyacrylamide gel electrophoresis (PAGE) was done according to Laemmli (12). Two-dimensional PAGE was carried out according to O'Farrell (13), as modified by Skene and Shooter (3), with 1.5-mm (diameter) tube gels containing 4% pH 3.5–10 and 2% pH 4–6 ampholytes.

Gels were stained with Coomassie brilliant blue (14). Destained gels were prepared for autoradiography by impregnating the gel with 2,5-diphenyloxazole (Sigma) according to the procedure of Jen and Thach (15), then drying under heat and reduced pressure, and exposure to XAR-5 x-ray film (Kodak) for 1–2 weeks at −70°C.

RESULTS

After injury to a peripheral nerve, cells in the nerve stump distal to the injury increase the synthesis and secretion of a 37-kDa protein, as assessed by *in vitro* labeling of the nerve stumps and collection of proteins released into the medium (3). Fig. 1 shows that, by 1 week after a sciatic nerve injury, the rate of *in vivo* synthesis of the 37-kDa protein is induced similarly compared to uninjured nerves and that this protein accounts for a major fraction of total protein synthesis in the distal nerve stump. Whether labeled *in vivo* or collected by *in vitro* incubation, the 37-kDa protein migrates on two-dimensional gels as a diagonal "comet-shaped" smear, suggesting considerable microheterogeneity. Previously, it has been shown that newly synthesized and secreted apoE migrates as a "smear" or as multiple, closely migrating bands reflecting a high degree of sialylation (16, 17).

Antibody Characterization. To probe for the specific classes of cells that secrete the 37-kDa protein and to further characterize the protein itself, we have purified the 37-kDa protein from injured peripheral nerves (8) and used this purified protein to generate a monospecific rabbit antiserum. The antibody specifically precipitates metabolically labeled 37-kDa protein from nerve-conditioned medium (Fig. 2 *Left*), establishing that the antibody, raised against $NaDodSO_4$-denatured material, recognizes the native protein. A 20-kDa polypeptide is also precipitated from conditioned medium under these conditions; this polypeptide is likely to be a fragment of the 37-kDa protein (see below). In electrophoretic transfer or immunoprotein blots, the antibody specifically recognizes the 37-kDa protein from medium conditioned by injured nerves (Fig. 2 *Right*) at dilutions up to 1:100,000. Barely detectable levels of the 37-kDa protein are present in conditioned medium from control nerves (Fig. 2 *Right*).

Injury of rat optic nerves also induces synthesis of a 37-kDa protein (3) that is similar in its two-dimensional electrophoretic profile to sciatic nerve-derived 37-kDa protein. Anti-37-kDa serum recognizes this protein (Fig. 2 *Right*), further indicating its homology to the sciatic nerve 37-kDa protein.

Cultured "Macrophage-Like" Cells Contain 37-kDa Immunoreactivity. Since homologous 37-kDa proteins are induced after nerve injury in the peripheral nervous system (PNS) and CNS, our efforts to locate the cells secreting the 37-kDa protein have included study of newborn rat brain cultures enriched for astroglia. Cultures 1–2 weeks old were treated with monensin to block secretion and allow intracellular accumulation of the 37-kDa protein. The monensin-treated cells were fixed and stained with antibodies to the 37-kDa protein and a fluorescent second antibody. In astroglial cultures the 37-kDa immunofluorescence was localized to a subpopulation of small cells (Fig. 3), which in control, single-labeling experiments do not stain with the astrocyte marker antiglial fibrillary acidic protein (data not shown). The presence of astroglia in these cultures was confirmed by

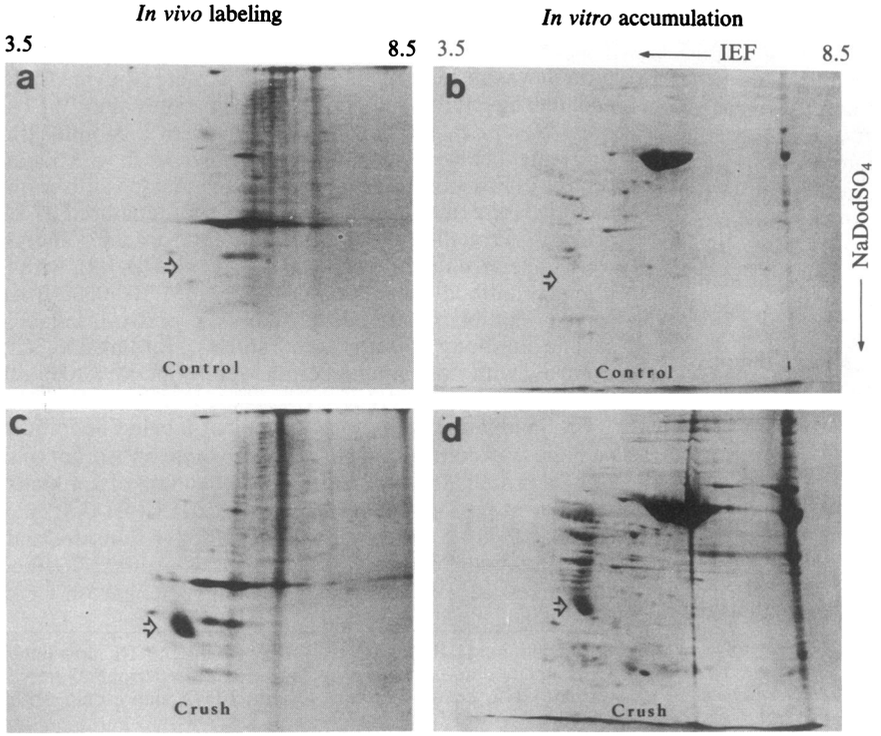

FIG. 1. Two-dimensional electrophoretic analyses of proteins present in the distal sheath of uninjured and crushed rat sciatic nerves. (*a* and *c*) Fluorographs showing the newly synthesized, $NaDodSO_4$-soluble proteins collected *in vivo* after direct injection of [^{35}S]methionine into the subperineurial space of control and crushed nerves. (*b* and *d*) Coomassie-stained gels of the accumulated proteins collected *in vitro* from equivalent segments of control and crushed nerves. Crushed nerves were examined *in vivo* 1 week after injury and *in vitro* nerves were examined 3 weeks after injury. In both instances, contralateral, uncrushed nerves served as controls. The arrow defines the position of the 37-kDa protein. IEF, isoelectric focusing.

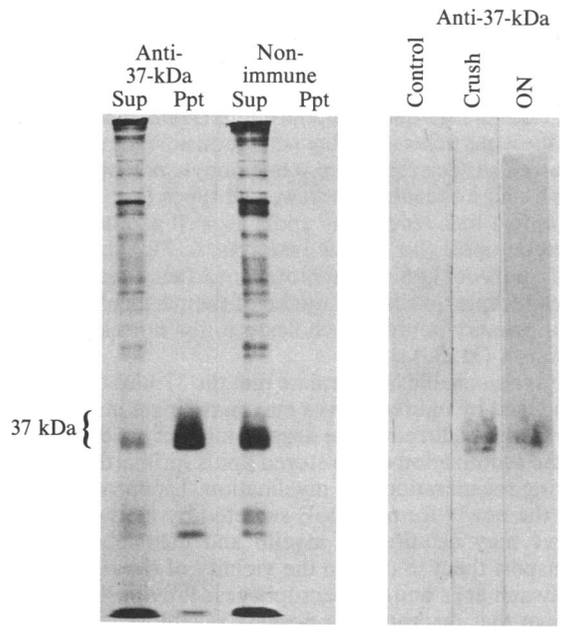

FIG. 2. Rabbit antibodies to the 37-kDa protein recognize the native and denatured 37-kDa proteins as well as the CNS-derived 37-kDa protein. (*Left*) Supernatants (Sup) and immunoprecipitates (Ppt) from samples of [^{35}S]methionine-labeled conditioned medium of 1-week post-crush nerves that were incubated first with antiserum to the 37-kDa protein or nonimmune serum for 4 hr at 4°C and then with protein A-Sepharose. Antiserum to the 37-kDa protein at 1:100 dilution precipitated the 37-kDa protein and a 20-kDa protein, a fragment of the 37-kDa protein. Nonimmune serum at 1:50 did not precipitate any labeled proteins. (*Right*) Immunoblots of unlabeled medium collected from control rat sciatic nerves (control), crushed sciatic (crush), and optic nerves (ON). At 1:10,000 dilution only the 37-kDa protein was recognized in crushed sciatic and optic nerve samples. Control nerves had little detectable immunoreactivity in this blot.

staining with antiglial fibrillary acidic protein (data not shown). Although the identity of these cells remains to be definitively proven, their morphology as well as their reactivity with antiserum to factor B (data not shown) suggest that they are macrophages or monocytes. Though several lines of evidence suggest that Schwann cells and astrocytes may be other sources of the 37-kDa protein *in vivo* (refs. 16, 18; unpublished observations), the immunoreactivity in macrophage-like cells under some culture conditions led us to compare the 37-kDa protein with the known secretory products of macrophages.

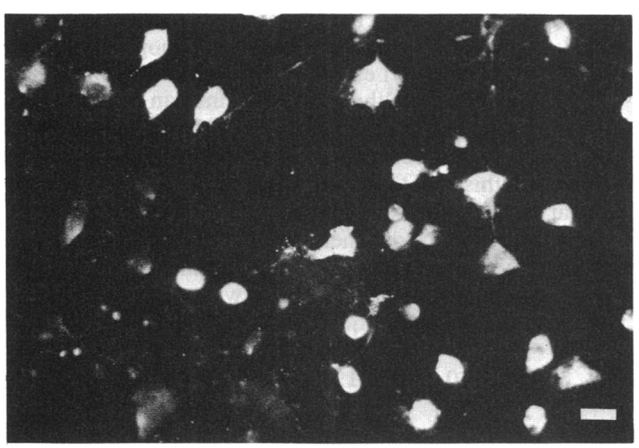

FIG. 3. Indirect immunofluorescence of astrocyte-enriched cultures stained with rabbit antiserum to the 37-kDa protein. Astrocytes prepared from neonatal rat cortex were fixed and stained. Before fixation, cultures were treated for 4 hr with 10 μM monensin to block secretion. Then, following fixation, anti-37-kDa serum was presented, followed by presentation of FITC-labeled goat anti-rabbit serum. With a FITC barrier filter in place, binding of the anti-37-kDa serum can be seen can be seen confined to a population of cells, 10 μm in diameter which coenrich with the astrocytes, that do not stain with antibodies to glial fibrillary acidic protein (not shown). (Bar = 10 μm.)

Identification of the 37-kDa Protein as apoE. Macrophages in culture can be induced to secrete apoE, a protein of similar molecular mass and isoelectric point (pI) to the 37-kDa protein from injured sciatic nerves (19, 20). Fig. 4 shows that antibodies raised against the sciatic nerve 37-kDa protein also recognize purified apoE. Conversely, antibodies raised against purified apoE also stain the nerve-derived 37-kDa protein. Since apoE occurs predominantly in serum and other extracellular fluids, we also probed normal rat serum with the anti-37-kDa antibody. As seen in Fig. 4 a serum protein similar in molecular mass to apoE was stained. The recognition of apoE by anti-37-kDa antibodies explains earlier findings that these antibodies recognize a serum protein (21) and our own finding that serum competes for binding of the 37-kDa antibody to electrophoretic transfer blots of sciatic nerve proteins (unpublished data).

Despite the clear antigenic similarity between apoE and the 37-kDa protein from sciatic nerves, electrophoretically discernible differences exist between these proteins. The injury-induced nerve protein recognized by both antibodies appears as a diffuse band that migrates more slowly than the most prominent band of either purified apoE or the presumed apoE in serum. The apparent higher molecular mass of the 37-kDa protein may reflect the high degree of sialylation of newly secreted apoE (16, 17).

Amino acid sequence analysis of the 37-kDa protein (purified as described in ref. 8) yielded two amino acids in

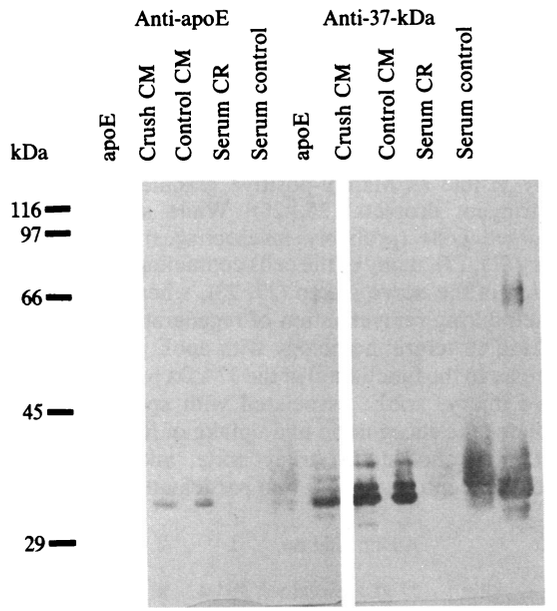

FIG. 4. Immunoprotein blots demonstrating the crossreactivity of antiserum raised against apoE and the 37-kDa protein for rat apoE, purified and in serum, and the 37-kDa protein in nerve crushed conditioned medium. Samples of apoE conditioned medium from 3-week "postcrush" nerve (Crush CM), control conditioned medium from uninjured nerve (Control CM), and serum from rats 3 weeks after crush of the sciatic nerve (Serum CR) or uninjured (Serum control) after separation in $NaDodSO_4$-containing 10% acrylamide gels were allowed to react with anti-apoE antiserum (*Left*) or anti-37-kDa antiserum (*Right*). Bound primary antibody was visualized with a horseradish peroxidase goat anti-rabbit antibody.

roughly equimolar amounts at each cycle through 14 cycles, as shown in Fig. 5. When these results were compared (Fig. 5) with the amino acid sequence of rat apoE (22), one set of amino acid residues corresponded to the N terminus of apoE and the other corresponded to an internal 20-kDa sequence that begins with aspartic acid at residue 145. This 20-kDa fragment was apparently generated during storage at −80°C, and evidence for a similarly sized fragment, immunoprecipitable with 37-kDa antiserum, can be seen in Fig. 2.

The match of 13 of 14 amino acids at the N terminus and an internal sequence of 14 amino acids along with the shared immunoreactivity are compelling evidence for the assertion that the 37-kDa protein is in fact apoE. The apparent molecular mass differences may reflect different degrees of glycosylation. However, the possibility of other posttranslational modifications of the protein accounting for the apparent molecular mass difference cannot be excluded.

DISCUSSION

By a variety of techniques we have shown that a 37-kDa protein induced after neuronal injury is homologous to a protein component of various serum lipoproteins, apoE. Both proteins have identical pI values between pH 5.3 and 5.5 (6, 7, 19, 20) and similar, though not identical, apparent molecular masses. Antibodies specific for the 37-kDa protein recognize apoE, and anti-apoE serum crossreacts with the 37-kDa protein. This indirect evidence for homology between the two proteins is further substantiated by direct comparison of partial amino acid sequences. Two fragments of the 37-kDa protein—13 of 14 amino acids at the N terminus and a 14-amino acid internal sequence—are identical to published amino acid sequences for corresponding regions of rat apoE (22).

A complex series of events occurs following nerve injury by crushing. During Wallerian degeneration of a heavily myelinated peripheral nerve, fragments of axonal and myelin membranes accumulate over the first 5 days after injury. These membranes are believed to be degraded by proteolysis, which disrupts protein–lipid interactions (23, 24). Lipids, including cholesteryl esters, accumulate in many cells of the distal nerve stump, particularly macrophages and Schwann cells, in the form of 0.5- to 2.0-μm lipid droplets, microscopically visible as Marchi-positive granules or sudanophilic, birefringent droplets (25, 26). While some of the lipid-engorged cells (probably macrophages) leave the site of injury (25, 27), many of the cells containing the esterified lipid remain in the nerve stump (27, 28), where the lipid may be reused during remyelination of regenerating axons (28, 29).

Close structural homology with apoE provides an important clue to the function(s) of the 37-kDa protein induced after nerve injury. apoE, associated with specific lipoproteins, mediates the recognition and uptake of lipoprotein particles containing cholesterol, triglyceride, and phospholipid by specific receptors on cells and participates in the redistribution of lipids among various cell types possessing the receptors (for review, see refs. 6, 7, 30). apoE is a major component of several serum lipoprotein particles, including chylomicron remnants, very low density lipoproteins or β-VLDL, and a subclass of high density lipoproteins (HDL-with apoE). apoE on these particles is the ligand responsible for recognition by two cell surface receptors, a hepatocyte receptor specific for apoE and a receptor on many cell types [the apoB,E(LDL) receptor] that recognizes apoE as well as another apolipoprotein, apoB (for review, see refs. 6, 7). Binding of apoE- and apoB-containing lipoproteins to these receptors stimulates receptor-mediated uptake of the particles, which serve as a primary source of cholesterol for normal cellular metabolism (31, 32).

It is reasonable to speculate that the 37-kDa (apoE) protein produced by injured nerves may participate in the storage of the lipids produced by the degeneration of the distal axon and in the reutilization of the stored lipids (primarily cholesterol) during regeneration and myelination. Lipoproteins possessing the newly formed apoE secreted by cells of the injured nerve may acquire the myelin and cell debris lipids and transport them to cells in the vicinity of the injury—e.g., to Schwann cells and/or macrophages. Previously, it has been shown that macrophages possess receptors capable of recognizing cholesterol-enriched lipoproteins containing the apoE and that the apoE on these lipoproteins is the ligand responsible for mediating the cellular uptake (33, 34). Uptake of these lipoproteins results in the accumulation of large quantities of stored cholesteryl esters. The droplets in these cells resemble the sudanophilic, Marchi-positive lipid droplets seen in cells during nerve degeneration. Furthermore, it has been shown that the cholesteryl esters stored in the macrophages can be mobilized from these cells when a suitable acceptor is present in the medium (33, 35). The most widely studied acceptor capable of acquiring the cholesterol is HDL. During this process of lipid mobilization the cholesteryl esters within the cells are hydrolyzed to free cholesterol, which is released from the cell. The cholesterol acquired by the HDL is then reesterified by the plasma enzyme lecithin:cholesterol acyltransferase. [It is noteworthy that the production of a similar enzyme is increased 5-fold within the tissue after injury to the rat sciatic nerve (36).] As the HDL become cholesteryl ester-enriched, they also acquire apoE, forming the HDL-with apoE. It has been established that apoE is required for these lipoproteins to be enriched in cholesteryl esters (33). In addition, by virtue of the presence of apoE, these lipoproteins can now be recognized by cells possessing the apoB,E(LDL) receptors and can participate in the redistribution of cholesterol from cells loaded with this lipid to those requiring cholesterol for normal cellular metabolism—e.g., biosynthesis of membranes.

Therefore, as axon degeneration occurs, the 37-kDa (apoE) protein may participate in mediating uptake of the lipids by Schwann cells and/or macrophages, where it could be stored until needed for regeneration. During regeneration, the apoE

Amino acid no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
37-kDa protein	Glu	x	Glu	Leu	Glu	Val	Thr	Asp	Gln	Leu	Pro	Gly	Gln	Ser	
apoE	Glu	Gly	Glu	Leu	Glu	Val	Thr	Asp	Gln	Leu	Pro	Gly	Gln	Ser	...
Amino acid no.	145	146	147	148	149	150	151	152	153	154	155	156	157	158	
37-kDa protein	Asp	Asp	Leu	Gln	Lys	Arg	Leu	Ala	Val	Tyr	Lys	Ala	Gly	Ala	
apoE	Asp	Asp	Leu	Gln	Lys	Arg	Leu	Ala	Val	Tyr	Lys	Ala	Gly	Ala	...

FIG. 5. Comparison of amino acid sequences of the 37-kDa protein with the known sequence for apoE. Thirteen of 14 amino acids can be aligned to match at the N termini of apoE and the 37-kDa protein as well as 14 of 14 amino acids in an internal fragment. The sequences shown were derived from a mixture of two fragments of the 37-kDa protein.

could participate in the redistribution of the cholesterol to neurons needing this lipid for axon growth and to Schwann cells for remyelination. This postulate is supported by the temporal sequence of events following nerve injury: accumulation of the 37-kDa (apoE) protein (3, 37), accumulation of lipid-laden cells (25), and presence of an increase in cholesterol-esterifying activity (23, 36). All three parameters begin to increase 4–5 days after nerve injury and reach their maximum by 3–4 weeks. In the CNS, synthesis of the 37-kDa protein is induced by nerve injury (3), but the accumulation of the protein is far less than in peripheral nerves (3, 5). A direct role for the 37-kDa protein in the clearance of axonal and myelin membrane debris might explain the slower time course of degeneration in the CNS, where degradation of myelin debris and formation of lipid storage droplets continue over a period of several months (38), and cholesteryl ester content does not change (39).

In addition to the possible role of the 37-kDa (apoE) protein in lipid transport and metabolism, it may participate in the regulation of other aspects of cellular metabolism within the degenerating and regenerating axon. For example, it has been shown that apoE interacts with specific receptors on lymphocyte membranes and renders these cells unresponsive to normal mitogenic stimulation (40, 41). Therefore, it is possible that the apoE regulates Schwann cell proliferation or other aspects of cellular metabolism, including axon growth.

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